The Role of Gonadal Sex Steroids in Neuroplasticity of Brains of Male *Peromyscus leucopus* Subjected to Different Photoperiods

Honors Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in Neuroscience in the undergraduate colleges of The Ohio State University

by

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Introduction

Day length can have major effects on brain and behavior. Neuroplasticity, the ability of the brain to reorganize through forming or eliminating different neural connections, can fluctuate throughout the year. For most animals, the time of year is determined internally based on day length (photoperiod). Male white-footed mice (*Peromyscus leucopus*) exposed to short winter-like day lengths have decreased hippocampal volume and fewer neuronal synapses when compared to those with long summer-like day lengths (Pyter, L.M., Reader, B.F. & Nelson, R.J., 2005). The hippocampus is a brain structure critical for regulating spatial learning and memory (Cassel J, Cassel S, Galani R, Kelche C, Will B, Jarrard L, 1998). These day length mediated (photoperiodic) changes in the structure and function of the hippocampus in *P. leucopus* are associated with impaired spatial learning and memory in short days. The mechanism for this neuroplasticity induced by photoperiod is not yet known.

Seasonal brain and behavioral changes may have its roots in the physiology and behavior of early ancestors. Maintenance of neuronal connections in the hippocampus is an energetically expensive process. The breeding season occurs during the long days of spring and summer for small mammals outside of the tropics; thus, individuals of many species are competing for limited resources. Outside of the tropics, short days signal winter when resources are scarce. The scarcity of resources in winter coincides with increased energetic requirements of thermoregulation. Because of the energetic challenges, small mammals rarely have sufficient reserves to breed during winter, and thus they have evolved adaptations, such as regression of their reproductive systems, potentially to conserve energy. During short days, *P. leucopus* act in more of a collective fashion, forming conspecific group nests to conserve heat energy (Workman, Bowers, & Nelson, 2009; King JA, 1968). It is possible that during the long days of

summer, males depend more on spatial learning and memory to compete for resources, such as breeding territories, and to successfully reproduce. Because male *P. leucopus* generally do not breed in the winter (King, 1968), they presumably do not require substantial spatial memory during short days, the short day reduction in hippocampal structure and function may have evolved to conserve energy (Pyter et al., 2005). Female *P. leucopus* are also seasonal breeders, but they are not responsible for maintaining a breeding territory, so their spatial learning and memory does not need to improve in long-days for reproductive purposes, as it does in males. Because females do not undergo a drastic change in their hippocampal-mediated behavior, spatial learning and memory tests based on day length are not generalizable to females (Yaskin VA, 2009).

Short days inhibit the hypothalamic-pituitary-gonadal axis, resulting in reduction of gonadal function to a prepubertal state (i.e., regressed size, decreased androgenesis and spermatogenesis) in photoperiodic rodents (Turek FW, Campbell CS, 1979), however, the mechanism by which short days decrease brain size and function is not obvious. Day length is encoded by the nightly duration of the secretion of melatonin from the pineal gland. A relatively long duration of melatonin secretion signals that outside is a long night (i.e., short-day), whereas a relatively short duration of melatonin secretion indicates a short night (i.e., long-day) (Carter & Goldman, 1983; Bittman & Karsch, 1984). Melatonin may influence neuroplasticity directly or indirectly in conjunction with gonadal sex steroids. However, because white-footed mice also regress their reproductive systems (and thus decrease circulating androgen and estrogen concentrations) in response to short days, it is possible that the low sex steroid hormone concentrations are provoking the brain and behavioral changes (Pyter et al., 2005; Workman, Bowers, & Nelson, 2009). If melatonin is directly influencing brain structure and function, then

these studies have more relevance to humans because humans with Seasonal Affective Disorder also change their melatonin in response to day length (Wehr, 2001). However, if sex steroid hormones are driving this change in brain and behavior, then it would suggest that photoperiod may not influence seasonal brain and behavior in humans because humans do not alter their reproductive systems in response to day length. My goal was to investigate a potential endocrine mechanism by which photoperiod affects hippocampal neuroplasticity and spatial learning and memory.

In this study I examined the interaction between sex steroids and day length. Sex steroid concentrations were varied by treating castrated male mice with implants of testosterone or its primary metabolites, estradiol (estrogen) or dihydrotestosterone (androgen). Each hormonal treatment group was exposed to either short or long day-lengths. I then measured hippocampal function in a spatial learning and memory task that has been validated in mice, the Barnes Maze.

Based on previous studies on the effects of androgens on spatial learning and memory in this species (Pyter LM, Trainor BC & Nelson RJ, 2006), I hypothesized that short day effects on spatial learning and memory performance, if mediated by changes in sex steroids, would be restored by androgens (testosterone or dihydrotestosterone), but not estradiol or cholesterol. It was unknown whether the effects of estradiol would mimic testosterone or oppose the behavioral effects of testosterone. Testosterone could either affect the hippocampus by acting via androgen receptors, or aromatize to estrogens and act on estrogen receptors. If testosterone were acting on the hippocampus after being aromatized to estradiol, then the effects of estradiol would instead mimic the effects of testosterone. Specifically, if the steroidal effects were mediated by androgen receptors, then short day mice exposed to testosterone or dihydrotestosterone should show reduced escape latency and fewer errors in the Barnes Maze compared to cholesterol- and

estradiol-treated groups. Alternatively, if short days impair spatial learning and memory independent of steroid treatment, then it may imply that the pineal hormone melatonin might directly affect photoperiodic neuroplasticity, instead of indirectly affecting neuroplasticity in conjunction with gonadal sex steroids.

Hypothesis: Short-day learning and memory deficits reflect low androgens which support spatial learning and memory.

Prediction: Androgen replacement will improve learning and memory in short days. Therefore, testosterone or one of its metabolites will improve learning and memory in short day mice to the degree of the long day mice.

Method

Animals and design.

One-hundred-twenty adult male white-footed mice (*Peromyscus leucopus*) were used in this study. This study utilized an analyisis of variance (ANOVA) design with eight different groups: bilateral castration plus cholesterol implant (CHOL; LD n = 15; SD n = 15), bilateral castration plus testosterone propionate implant (CAST + T; LD n = 15; SD n = 15), bilateral castration plus dihydrotestosterone implant (CAST + DHT; LD n = 15; SD n = 15), and bilateral castration plus estradiol benzoate implant (CAST + E; LD n = 15; SD n = 15). Cholesterol is the metabolic precursor of all of the hormones above and served as a control group.

Surgery and photoperiod manipulation.

Under deep anesthesia using sterile surgical techniques, mice underwent bilateral castration surgery. The mice were treated with 5% isoflourane, then kept under deep anesthesia with 2% isoflourane during the gonadectomy. I shaved the fur from a one square inch abdominal section and a one square inch midscapular section. The abdominal area was scrubbed in a circle with betadine. I cut 1cm at the midline and externalized the testes. The testicular arteries were cauterized and the epididymis, fat tissue, and vas deferens were replaced into the abdominal cavity. The abdominal wall and skin were sutured back up with sterile nylon sutures. The abdominal muscle was sutured in an interrupted suture technique using sterile 6/0 adsorbable suture, and the skin was sutured using an interrupted suture technique using 6/0 nylon suture. A 1cm incision was made at the midscapular surgical site and a sealed Silastic tube implant containing either estradiol benzoate, dihydrotestosterone, testosterone propionate, or cholesterol (10mm length; soaked in saline 2 h prior to implantation) was implanted under the skin. Implants were designed to be similar to that of long-day photoperiodic concentrations of steroids. The skin was sutured with 6/0 sterile nylon suture. The mice were somewhat randomly assigned to either remain in long days (LD; 16h light:8h dark) or short days (SD; 8h light:16h dark) for ten weeks, a time shown to induce maximal reproductive responses to photoperiod in this species.

Spatial Learning and Memory Testing

The Barnes Maze tested learning and memory by exposing mice to a circular platform with 20 equally spaced holes along the perimeter. Only one of the holes, the target hole, had an escape box beneath it where the mice could seek shelter hide. The mice were negatively reinforced by the exposure on the open platform and a bright light, and positive reinforcement was provided by the dark escape box. Visual cues such as different colors and shapes were also placed around the maze. The mouse was allowed to explore the maze for three minutes, during

which total errors and latency to escape are recorded by a video tracking system. When the mouse entered the escape box, the mouse was allowed to stay in it for one minute. The mice received three trials a day with an inter-trial interval of fifteen minutes during across five days of training. On the sixth day, a probe trial was conducted in which a mouse was allowed to explore the maze for ninety seconds to determine if the mouse remembered where the target was located based on visual cues. However, the maze during the probe trial was different from the training days in that the target box was covered so that the mice could not escape into it. Learning and memory was tested in this way by measuring how long it took a mouse to learn where the escape box hole was located on training days (learning), and how much time it spent in the vicinity of the escape box location during probe trials (memory retention). Mice that were significantly impaired in learning and memory have a greater latency to escape and made more errors during training and spent less time in the vicinity of the target hole and made more errors during probe trials.

Tissue collection and RNA Extraction

Twenty-four hours after the conclusion of behavioral testing, mice were killed and I dissected out the seminal vesicles and brains and weighed them, Brains were then preserved in RNAlater and stored at -70°C. To extract RNA, I cut the cerebrum at the midsagittal fissure then dissected out one hippocampus. RNA was extracted with Trizol using a homogenizer (PowerGen 1000). Extracted RNA was suspended in 30 μL RNase-free water. A spectrophotometer (NanoDrop 1000) was used to detect any Trizol contamination and determine RNA concentration. For each sample, 2μg of RNA was reverse-transcribed into cDNA using MMLV Reverse Transcriptase enzyme (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol.

qPCR

In order to determine why estrogen and testosterone had opposite effects on spatial learning and memory depending on photoperiod, we tested for the presence of β-estrogen receptor (ERβ), α-estrogen receptor (ERα), and androgen receptor (AR), gene transcripts from the hippocampi of the mice using rtPCR and the following primers and probes and probes designed for *P. leucopus* (after Pyter et al., 2006):

ERα forward 5'-GAACAGCCCCGCCTTGT-3'
ERα reverse 5'-GCATCCAGCAAGGCACTGA-3'
ERα probe 5'-TGACAGCTGACCAGATG-3'
ERβ forward 5'-GCTGATGTGGCGCTCGAT-3'
ERβ reverse 5'-CCCTCATCCCTGTCCAGAAC-3'
ERβ probe 5'-ACCACCCTGGCAAGCTCATCTTT-3'
AR forward 5'-GTGGTGTGTGCTGGACATGAC-3'
AR reverse 5'-GGCTAGATAACAGGGCAGCAA-3'
AR probe 5'-ACAACCAACCTGACTCC-3'

A TaqMan 18S Ribosomal RNA primer and probe set (labeled with VIC; Applied Biosystems) was used as the control gene for relative quantification. Amplification was performed on an ABI 7500 Fast Real-Time Sequencing by using Taqman Universal PCR Master Mix. The universal two-step RT-PCR cycling conditions used were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. Relative gene expression of a duplicate individual samples was calculated by comparison to a relative standard curve. Relative gene expression of individual samples run pseudo-randomly in duplicate was calculated by comparison to a relative standard curve consisting of serial dilutions of pooled *P. leucopus*

hypothalamic cDNA (1:1, 1:10, 1:100, 1:1000) followed by normalization to 18S rRNA gene expression.

Statistical Analyses

Repeated measures ANOVAs, which is used to compare means of multiple groups when the same mice are subjected to repeated measurements, were used to compare Barnes Maze performance over training days with photoperiod and steroid treatment as variables. MANOVA, which compares means of groups when different mice were used in each group, was used to compare steroid and photoperiod-dependent behavior during the probe trial. Comparisons between estrogen and testosterone were planned *a priori* (Pyter et al., 2006 & Trainor BC, Lin S, Finy MS, Rowland MR & Nelson RJ, 2007). A *t*-test, which compares means between two groups, was used to compare seminal vesicle mass between photoperiods, and ANOVA was used to compare seminal vesicle means with steroid treatment. MANOVA was also performed on the PCR data to examine gene expression levels of steroid receptors during SD and LD. All comparisons were considered statistically significant when $P < 0.05$. IBM SPSS was used for all analyses.

Results

Reproductive Measures

I weighed the seminal vesicles in order to ensure that the Silastic tube implants of testosterone, dihydrotestosterone, estradiol, and cholesterol were working in long days and short days for the entirety of the study. Photoperiod did not affect seminal vesicle mass (t_{35}=0.008, p=0.93), but there was an effect of steroid treatment on this measure ($F_{3,49}$= 157.800, p<0.05; Figure 1). The results verified that the implants delivered steroids across the study because the

reproductive structures were largest for testosterone and decreased with dihydrotestosterone, estradiol, and cholesterol, respectively.

Barnes Maze

In order to test the effects of gonadal sex steroid concentrations and photoperiod on neuroplasticity, we manipulated estradiol, testosterone, dihydrotestosterone, and cholesterol in *P. leucopus* within short-day and long-day groups and tested spatial learning and memory using the Barnes Maze. All sex steroids combined and photoperiod did not differ in errors to find the target hole ($F_{(3,25)}$=1.382, p=0.271; Figure 2) or latencies ($F_{(3,25)}$=0.983, p=0.456) across training days. Similarly, neither errors to the target hole ($F_{(3,26)}$=1.416, p=0.261) nor latencies ($F_{(3,26)}$=0.996, p=0.410) differed significantly among all sex steroids combined and day length during the probe trial. However, we made an *a priori* decision to compare just estradiol and testosterone in SD and LD. Previous research showed that testosterone improved spatial learning and memory measures in SD (Pyter et al., 2006). I needed to compare testosterone and estrogen in order to determine whether testosterone was mediating this neuroplasticity, or if it was being aromatized to estrogen before affecting the hippocampus. I found significant differences when comparing the errors and latencies of just estradiol and testosterone. Estrogen and testosterone had opposing effects on errors and latencies depending on photoperiod. Mice receiving estrogen made fewer errors in long days and those receiving testosterone made fewer errors in short days. The effects for latencies were similarly opposite as well. Latency to escape was shortest in short days for estrogen and shortest in long days for testosterone. Estradiol and testosterone alone had an interaction with photoperiod on training errors ($F_{1,25}$=4.825, p<0.05; Figure 3 and 4) and probe errors ($F_{1,23}$=4.176, p=0.052; Figure 5 and 6). Likewise, estrogen and testosterone interacted with photoperiod to affect probe latencies ($F_{1,23}$=4.26, p<0.05; Figure 7 and 8). So the estradiol and

testosterone affected the errors and latencies to the target hole differently depending on which photoperiod the mice were in. Therefore, spatial learning and memory depends on the presence of testosterone and estradiol and photoperiod. Estradiol improved learning and memory in long days and testosterone improved learning and memory in short days. The enhanced spatial ability by testosterone is short days was expected from the hypothesis based on previous studies (Pyter et al., 2006). The finding for estradiol and photoperiod is a novel finding and suggests that testosterone is rescuing hippocampal deficits in SD instead of being aromatized to estradiol because estradiol did not improve spatial learning and memory in short days. Had estradiol and testosterone had the same effects in SD, then we could have said testosterone was improving hippocampal functioning by being aromatized to estradiol. Photoperiod and sex steroids interacted to affect errors to get to the target hole and latency before reaching the target hole in the Barnes maze throughout the 5 day trial and during the probe trial only when comparing estradiol and testosterone.

Gene Expression Assays

To explain the underlying mechanism of the photoperiod-steroid interaction, I conducted a gene expression assay on brain tissue from long-day and short-day *P. leucopus*. We did not find any effect of treatment for AR ($F_{(3,29)}$=2.680, p=.065,Figure 9), ERα ($F_{(3,29)}$=2.341, p=.094, Figure 10), or ERβ ($F_{(3,29)}$=1.663, p=.197, Figure 11) depending on photoperiod and steroid presence. Although not to the level of significance, AR gene expression was higher in the SD group for testosterone, and ERα and ERβ gene expression was higher in the LD group for estrogen (Figure 12). However, there was a difference in AR gene expression for cholesterol between LD and SD ($F_{7,29}$=1.274, p=0.052). This shows that when no steroids are present, there are different levels of expression for AR in different photoperiods.

Discussion

Estradiol and testosterone have opposing effects on spatial learning and memory depending on the photoperiod. Estradiol enhanced spatial learning and memory in long days and testosterone enhanced spatial learning and memory in short days. Therefore, day length and steroids interact to alter spatial learning and memory. The effects of testosterone and dihydrostesterone on spatial learning and memory in the current study were consistent with previous studies. However, in previous studies, it was not clear whether testosterone was affecting spatial learning and memory as an androgen, or if it was acting on the hippocampus by being metabolized to estradiol.

In this study I tested spatial learning and memory with steroids present under a photoperiod manipulation, but previous research has reported on the effects steroids have on the hippocampus when photoperiod has not been taken into consideration. Testosterone maintained normal levels of synaptic spine density in the male hippocampus, showing that it is necessary for normal spatial learning and memory levels in males (Leranth C, Petnehazy O & Maclusky NJ, 2003). This study also suggested that testosterone is acting via AR because it was treated with non-aromatizable testosterone. Another study showed that treating female Sprague-Dawley rats with testosterone increased spatial learning and memory ability to the level of male rats, which is naturally better then female performance (Roof RL & Havens MD, 1992). When an androgen-inhibitor was given to *P. leucopus*, spatial learning and memory was impaired (Pyter et al., 2006). However, the literature on the effects of estrogens on the hippocampus is inconsistent

among species. The presence of estradiol also improves synaptic connections in the hippocampus, and treatment with an aromatase-inhibitor (which prevents testosterone from being aromatized into estradiol) decreases synaptic connections and spatial learning and memory in rats (Prange-Kiel J, Fester L, Zhou L, Lauke H, Carrétero J & Rune GM, 2006). However, in Sprague-Dawley rats, estradiol did not seem to have an effect on the hippocampus (Leranth C et al., 2003). So whereas testosterone seems to consistently enhance spatial learning and memory, the effects of estradiol on the hippocampus in static lighting conditions are less clear.

The behavioral effects from testosterone occur when testosterone either binds directly to androgen receptors, or when is metabolized to estradiol or dihydrotestosterone, which then bind to their respective receptors, ERα/ERβ and AR. I expected the behavioral differences in estradiol and testosterone to be due to differing numbers of receptors depending on photoperiod. In California mice, estrogen decreased aggression in long-days and increased aggression in short-days (Trainor BC, Bird IM & Marler CA, 2004). They investigated this mechanism by looking at the expression of ERs. ERα increased in SD, and ERβ increased in LD. Estradiol acted rapidly in the bed nucleus of the stria terminalis in *Peromysucs polionotus* to increase aggression in LD, which gave evidence of a nongenomic mechanism (which acts in minutes and regulates gene expression without binding to DNA), but estradiol acted slowly to decrease aggression in SD, which gave evidence of a genomic mechanism (which acts in hours/days and regulates gene expression by binding to DNA). In addition, the differences in aggression in this study was not due to changes in receptor expression in the hippocampus depending on day length, which suggests that day length can affect how receptors function without changing their relative expression. (Trainor et al., 2007). This emphasizes how aggression is mediated by neurobiological factors under different environmental signals. Another study that tested the effects of

testosterone on spatial learning and memory in different photoperiods had similar behavioral results for testosterone as the current study, but they did not find differences in ARs or ERs with photoperiod (Pyter et al., 2006). However, the mRNA that was examined for gene expression did not come from the animals that were tested and treated with testosterone.

In order to examine the mechanism for the different behavioral effects of estradiol and testosterone depending on photoperiod, I did a gene expression assay for AR, ERα, and ERβ. However, there were no significant differences due to photoperiod in receptor expression in the presence of different steroids. The trends based on the graph did show that ARs were more abundant in the SD group when testosterone was present, which parallels the results of the behavioral assay, given that testosterone improves spatial learning and memory in SD. Similarly, ERα and ERβ were more abundant in the LD group when estrogen was present, and estrogen improves spatial learning and memory in LD. There also was a significant different number of AR in the cholesterol group, when no gonadal steroids were present, in SD and LD, indicating that photoperiod alone changed the expression of AR at some level. However, these gene expression results in the E and T groups were not statistically significant, most likely due to a low sample size and large variance. In the future, there will be more mice added to this study to see if these trends reach significance. If these data prove to be significant with larger sample sizes, then it will confirm that estradiol and testosterone improve spatial learning and memory in their respective photoperiods due to a differential expression of their receptors.

Our results demonstrate that testosterone increases spatial learning and memory in short days and estrogen increases spatial learning and memory in long days, thus they support the hypothesis that the mechanism of neuroplasticity dependent on photoperiod is due, at least in part, to fluctuating steroid concentrations. The hippocampus responds to sex steroids differently

depending on day length. These findings relate back to the fact that *P. leucopus* breed in the summer. When they breed they need to use their hippocampus more for territoriality in order to increase survival probability of their young. *P. leucopus* do not generally reproduce in the winter, so they do not need an enhanced hippocampus to maintain breeding territories. Because reproduction and enhanced hippocampus function are energetically expensive, it is adaptive for them to decrease these functions in the harsh short days of winter. This research suggests that the changing day length along with fluctuating gonadal sex steroids mediates this neuroplasticity of the hippocampus.

These results may have implications for disorders that come about in certain seasons. For example, seasonal affective disorder (SAD) is a disorder that inflicts people with depression in the winter. If the mechanism that drives the reduction of hippocampal functioning in SD is discovered, then there may be insight to what is driving SAD and other seasonal disorders in humans.

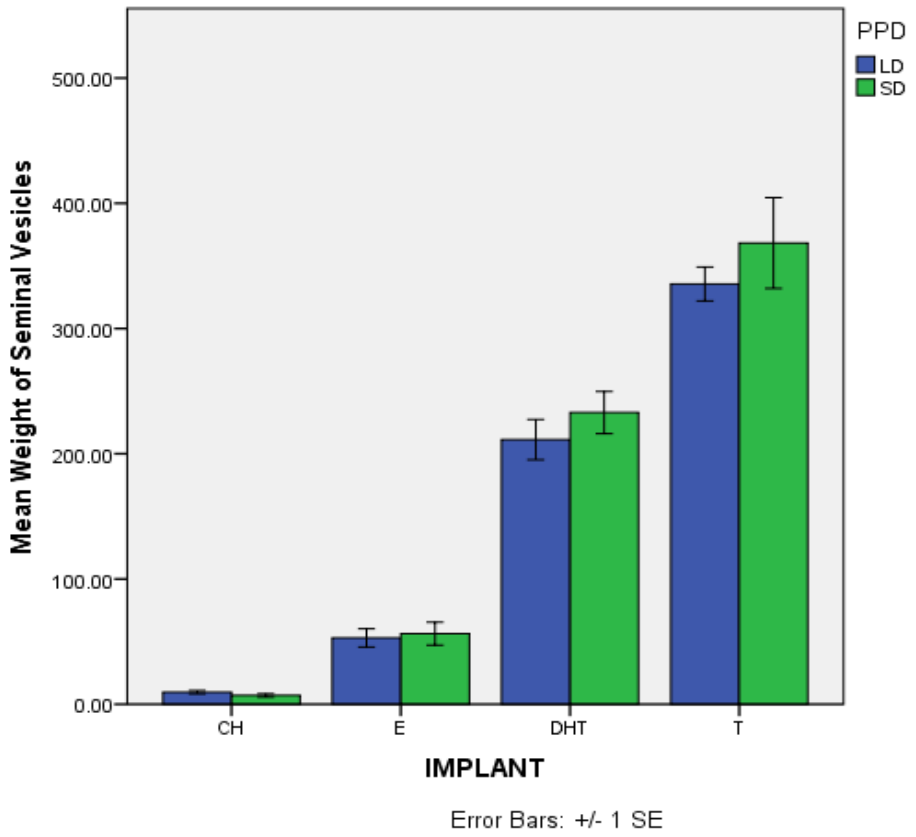

Figure 1: The weights of the dissected seminal vesicles show that the Silastic Tube implants successfully secreted sex steroid throughout the study. The seminal vesicles were maximally reactive to testosterone and dihydrotestosterone and minimally reactive to cholesterol and estrogen, which is similar to how endogenous steroids would have affected them.

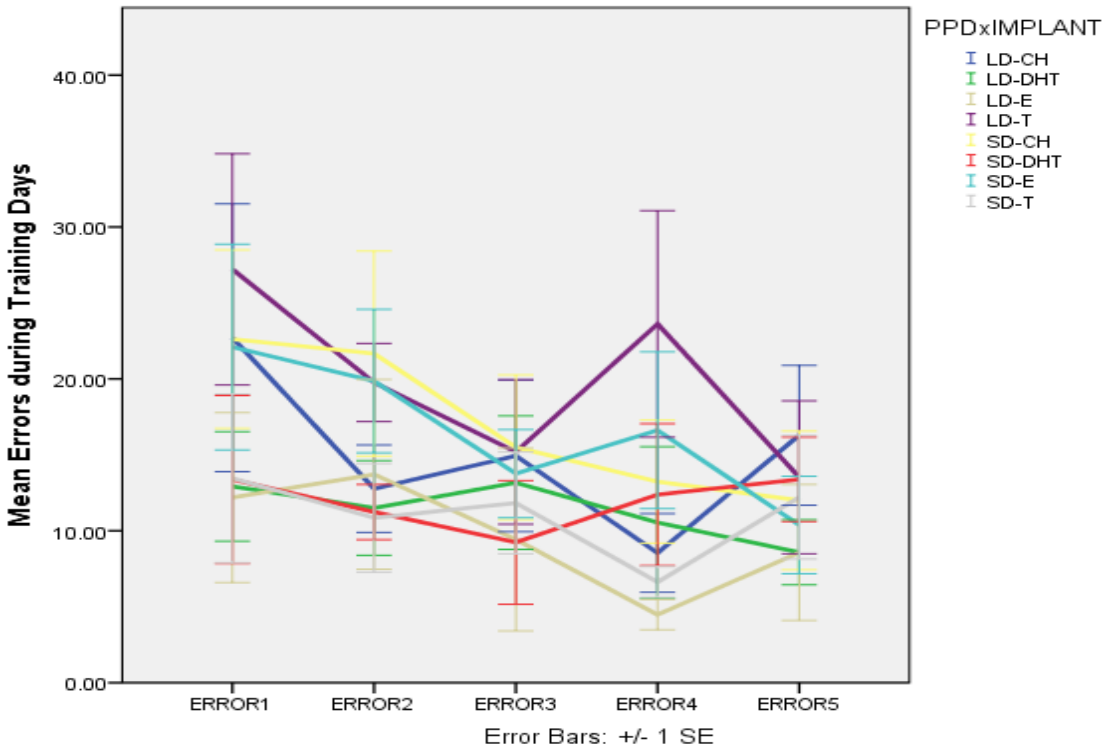

Figure 2: There were no significant differences of errors to the target hole during training days when examining all sex steroids in both LD and SD.

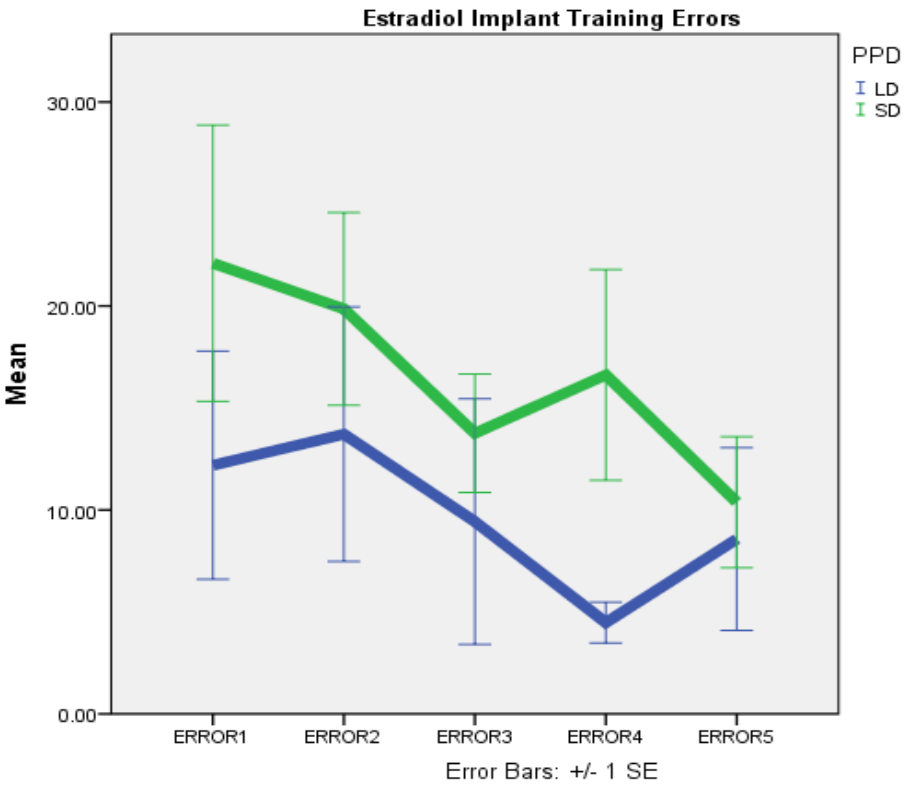

Figure 3: Estrogen-treated mice made fewer errors prior to reaching the target hole in LD compared to SD, thus estrogen enhances spatial learning and memory in LD.

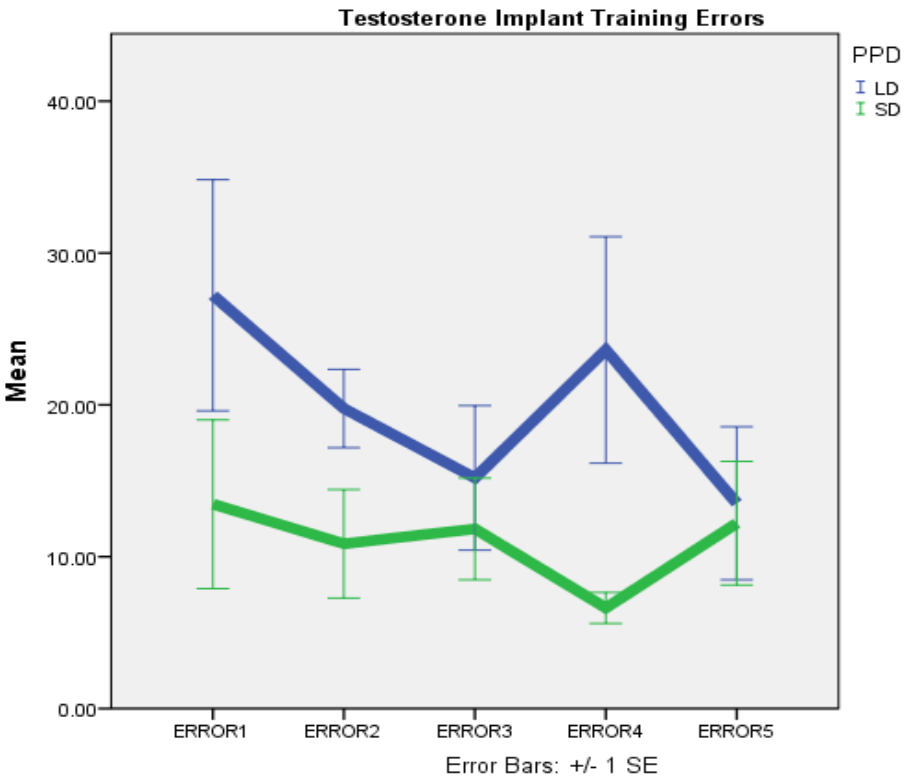

Figure 4: Testosterone-treated mice made fewer errors to the target hole in SD in comparison to LD, thus testosterone enhances spatial learning and memory in SD.

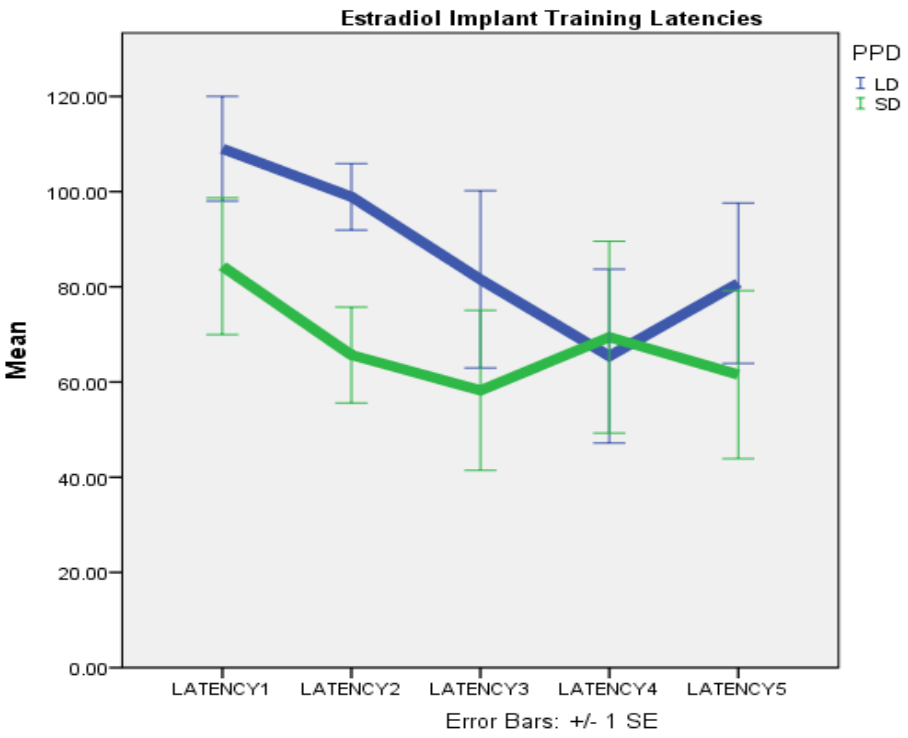

Figure 5: Estrogen-treated mice had a lower latency to the target hole, thus took less time to get to the target, in SD.

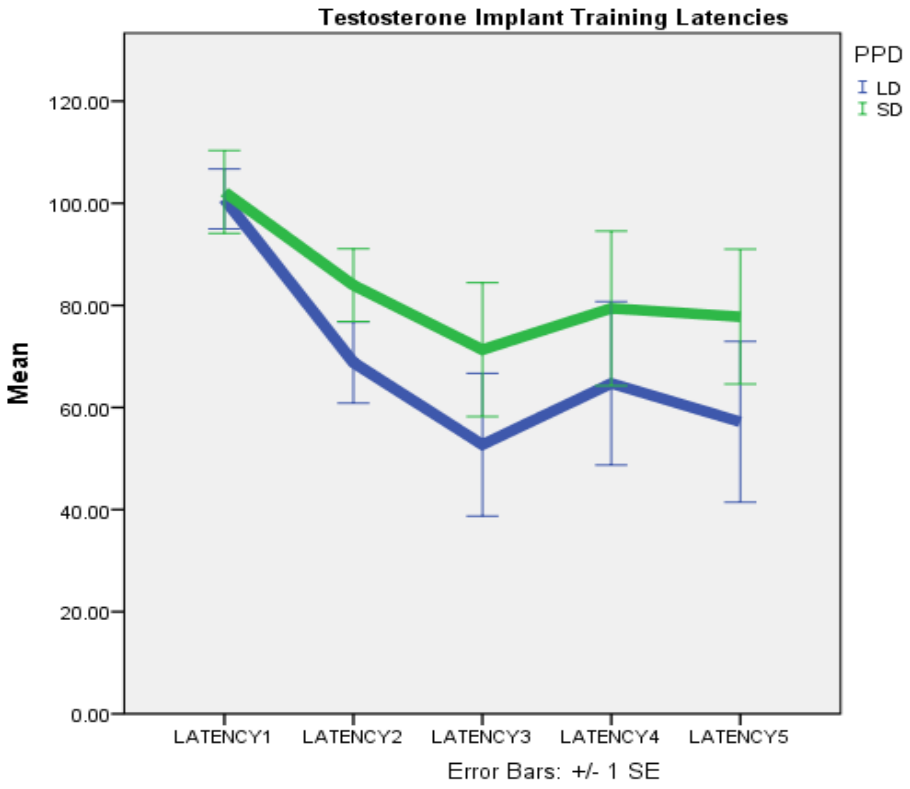

Figure 6: Testosterone-treated mice had lower latency to the target hole, thus took less time to get to the target, in LD.

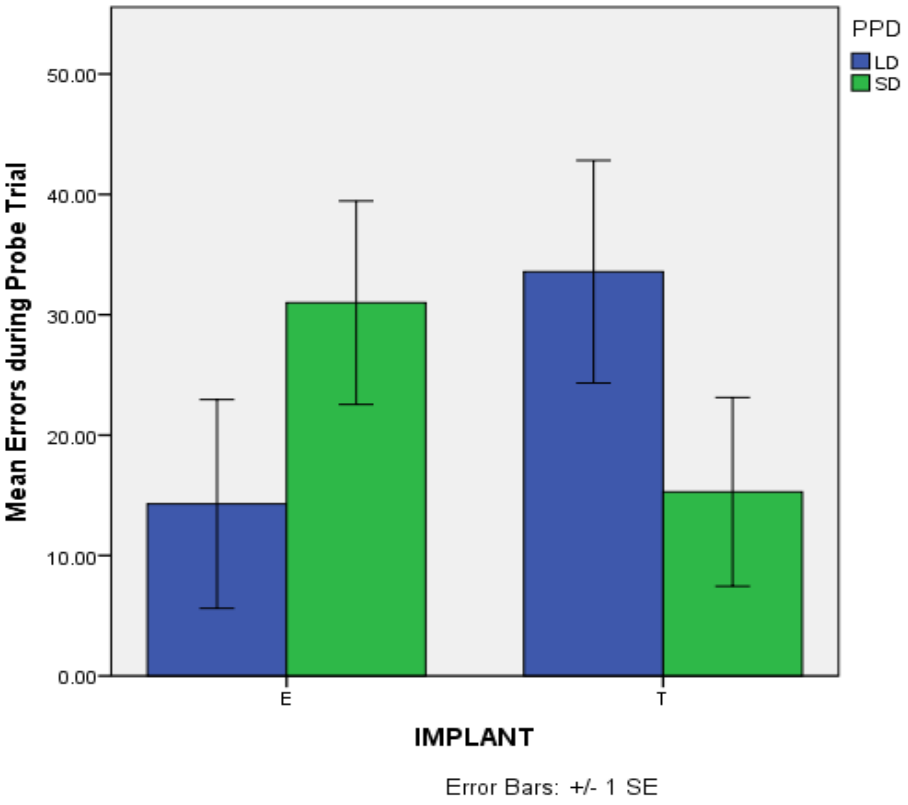

Figure 7: Estrogen and testosterone interact with day length to affect errors in the probe trial. Mice receiving estrogen made fewer errors in LD, and mice receiving testosterone made fewer errors in SD, as was shown in training days.

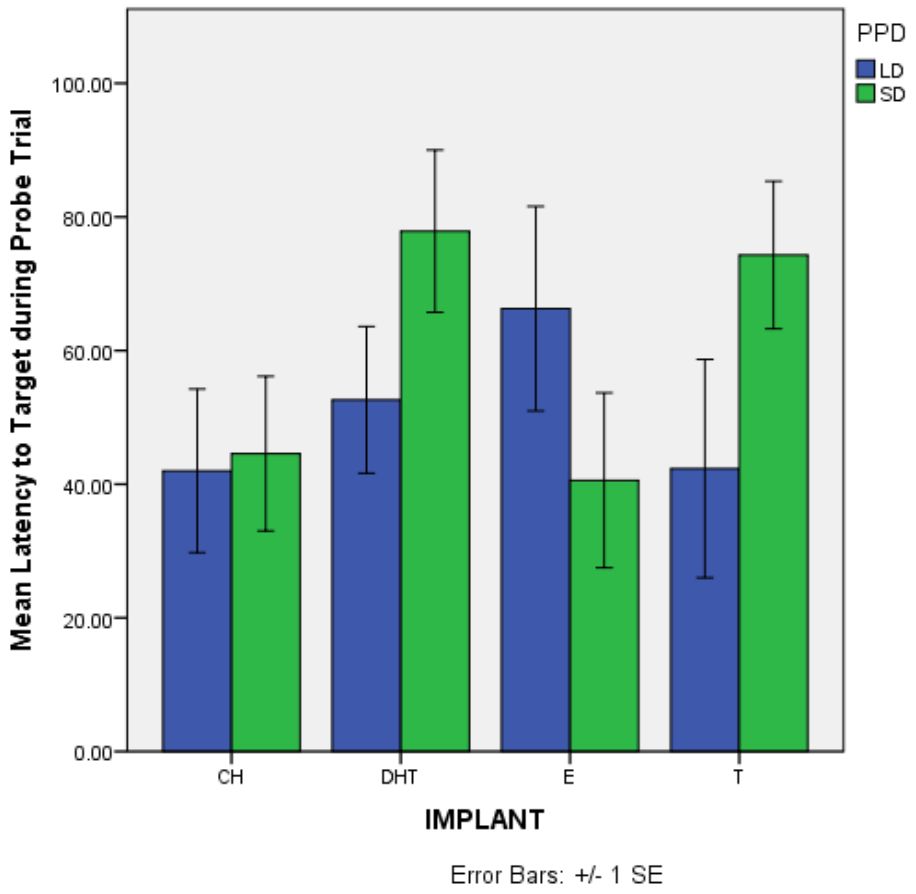

Figure 8: Estrogen and testosterone showed opposite latency trends depending on photoperiod. Dihydrotestosterone mimicked testosterone, and cholesterol was fairly constant among photoperiod.

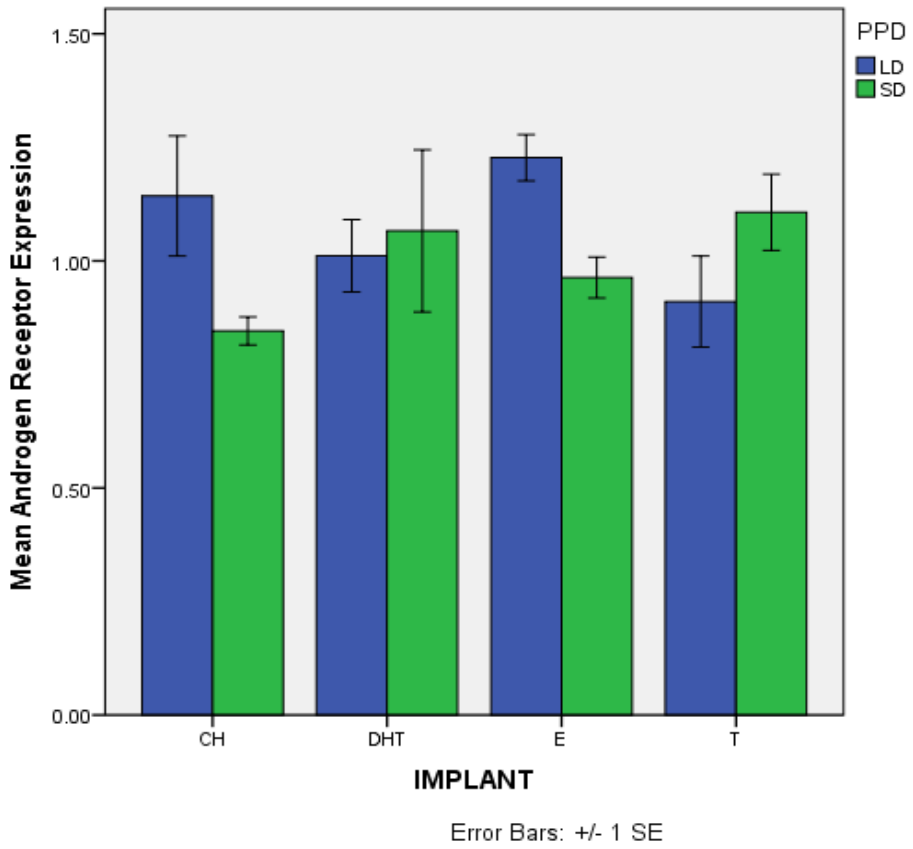

Figure 9: Although not to the level of significance, androgen receptors are more abundant in SD when testosterone is present. This mimics the fact that testosterone improves learning and memory in SD.

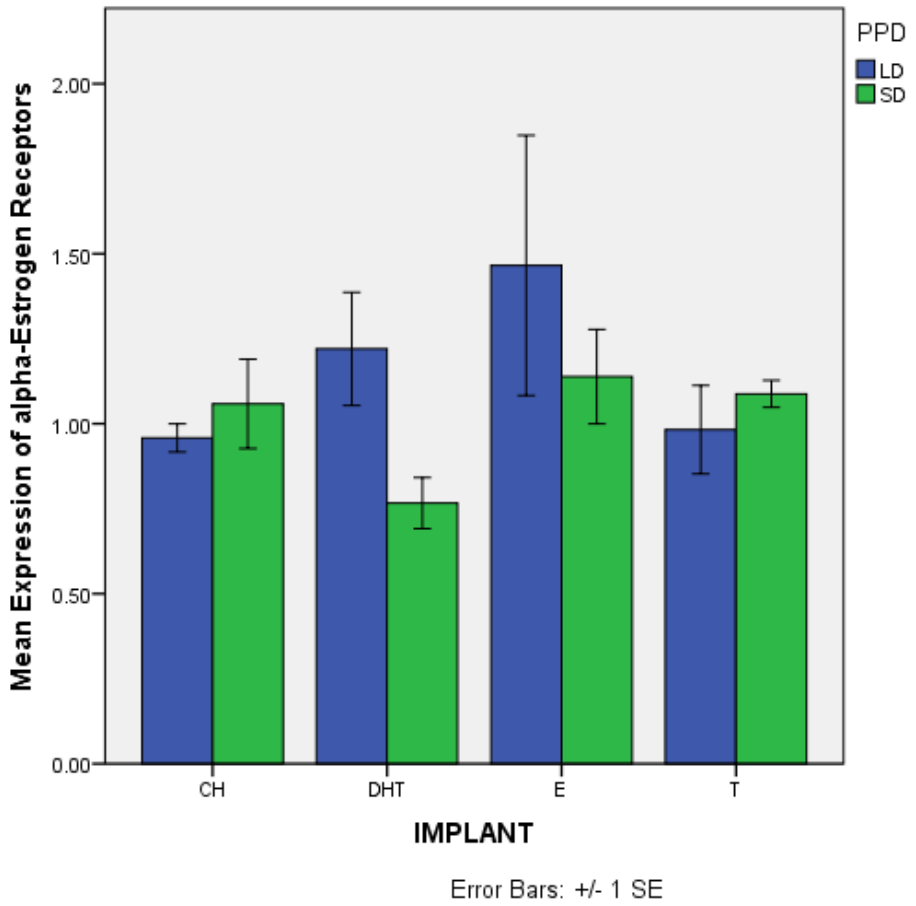

Figure 10: Although not to the level of significance, ERα receptors are more abundant in LD when estrogen is present. This mimics the fact that estrogen enhances spatial learning and memory in LD.

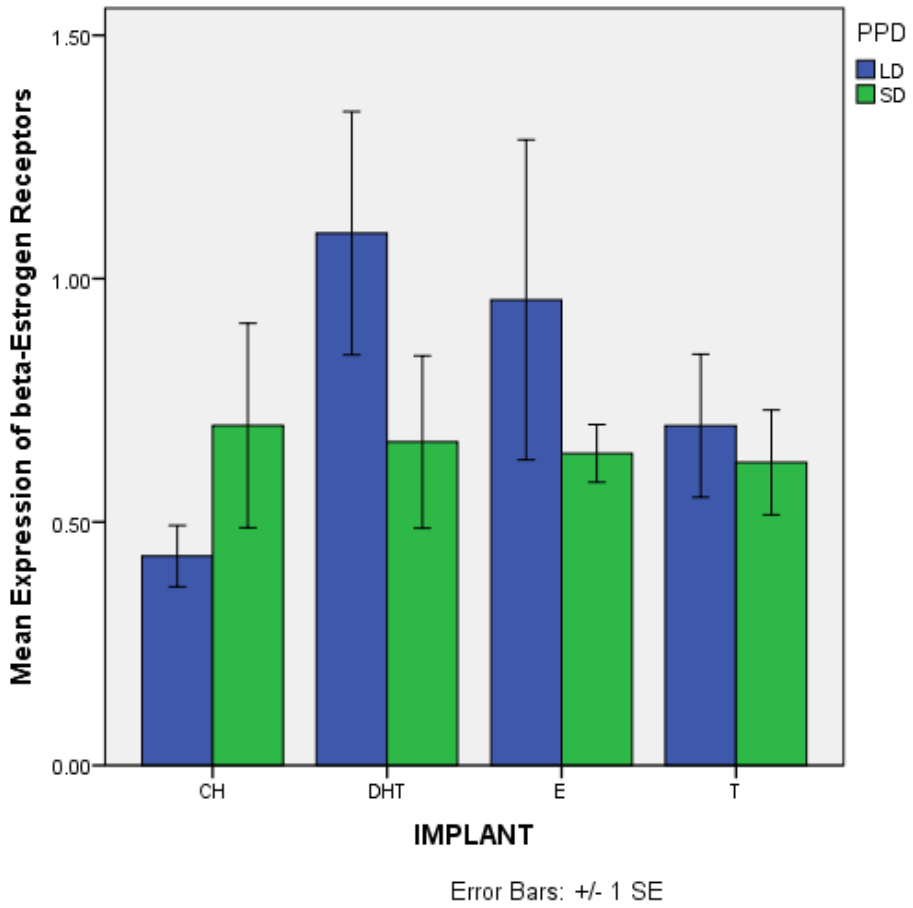

Figure 11: Although not to the level of significance, ERβ receptors are more abundant in LD when estrogen is present. This mimics the fact that estrogen enhances spatial learning and memory in LD.

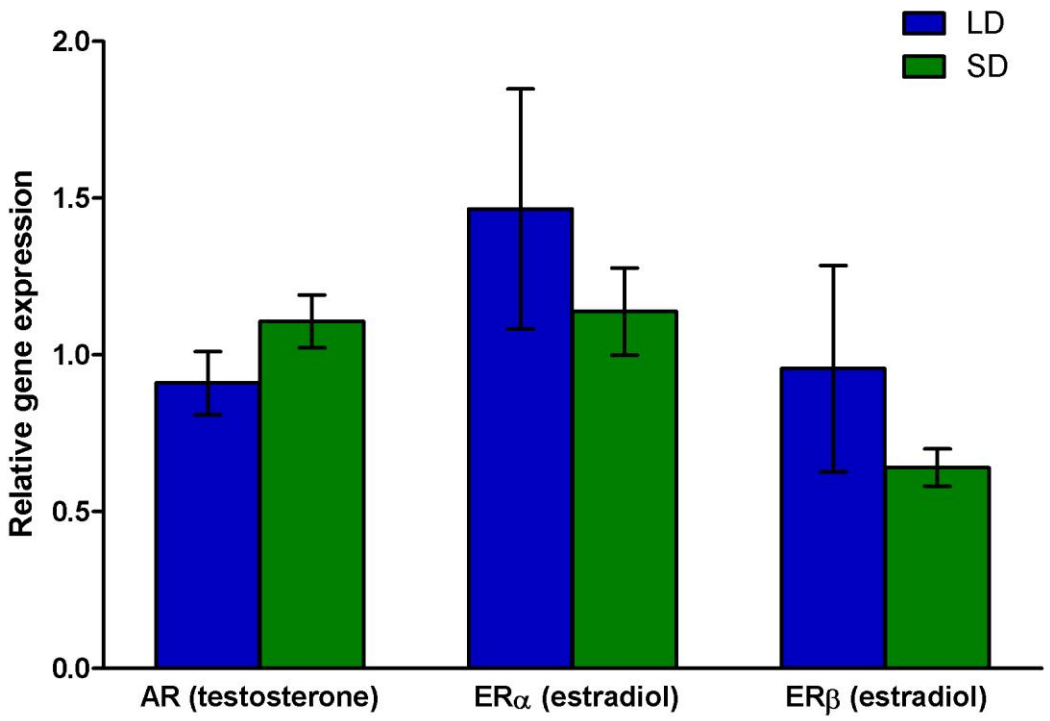

Figure 12: Androgen receptors are more abundant in SD when testosterone is present, and ER are more abundant in LD when estrogen is present. Although not to the level of significance, receptor expression trends mirror behavioral trends in Barnes maze errors.

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